Studies of Lysosomal α-Glucosidase. II. Kinetics of Action of the Rat Liver Enzyme*

Peter L. Jeffrey,† David H. Brown, and Barbara Illingworth Brown‡

ABSTRACT: The kinetics of action of a purified α-glucosidase from rat liver lysosomes has been studied using oligosaccharide and polysaccharide substrates. Maltose, maltotriose, maltotetraose, and maltopentaose all exhibit strong substrate inhibition at concentrations greater than approximately 8 mM. Neither glycogen nor isomaltose shows such inhibition. Isomaltose is a competitive inhibitor of maltose hydrolysis. The K_m of glycogen and the V_{max} of α-1,4-glucosidase action on it are both increased about nine times by increasing the cation concentration at pH 4.2 from 4 to 115 mM. Mutual inhibition studies using maltose and glycogen have indicated that each substrate appears to be a competitive inhibitor of the other. However, differences in response to cation concentration and to pH suggest the possibility that the enzyme has more than one catalytically active binding site as well as at least one separate inhibitory site. The existence of the latter site is indicated by the substrate inhibition results obtained with oligosaccharides. D-(+)-Turanose has been found to give mixed inhibition of the hydrolysis of all substrates investigated. With maltose, the inhibition appears to be partially competitive plus purely noncompetitive in type. With glycogen, inhibition is almost purely noncompetitive. With isomaltose, the nature of the reciprocal plots in the presence of turanose depends strongly upon the turanose concentration. These results support the suggestion that the enzyme has multiple substrate binding sites. The lysosomal α-glucosidase is able to catalyze the total hydrolysis of glycogen to glucose. Debranching of the polysaccharide in the course of its hydrolysis is the rate limiting step in this overall process.

The preceding paper (Jeffrey *et al.*, 1970) described the purification and properties of an α-glucosidase from rat liver lysosomes. The enzyme was shown to have activity both as an α-1,4-glucosidase and as an α-1,6-glucosidase. The same protein is active in the transglucosylation of oligosaccharides and polysaccharides. The present paper describes kinetic studies of the action of this enzyme on various oligosaccharides and on glycogen. The lysosomal α-glucosidase is shown to be able to catalyze the total hydrolysis to glucose of glycogen and of polysaccharides of related structure.

Materials and Methods

Maltose, uniformly labeled [^{14}C]maltose, isomaltose, and glycogen were obtained from commercial sources and purified as described in the preceding paper (Jeffrey *et al.*, 1970). D-(+)-Turanose was obtained from Mann Research Laboratories. Maltotriose, maltotetraose, and maltopentaose were purchased from Pierce Chemical Co., and before use they were purified by paper chromatography as described for other oligosaccharides in the preceding paper.

[^{14}C]Glycogen was prepared by fasting a 3-kg rabbit for 48 hr and then injecting it intraperitoneally with 2 g of glucose as a 20% solution in 0.9% NaCl. One hour later 1 ml of a solution containing 28.6 mg of uniformly labeled [^{14}C]glucose (6.3 mCi/mmole) in 0.9% NaCl was injected intravenously. Six hours after the second injection the animal was anesthetized with Nembutal. Following exsanguination by cardiac puncture, the liver was perfused with isotonic saline, and a protion of the liver tissue then was digested in two volumes of 30% NaOH for 3 hr at 100°. The glycogen was precipitated with ethanol and then was reprecipitated five times from aqueous solution. The final preparation was exhaustively dialyzed against distilled water. The [^{14}C]-glycogen (3110 cpm/μmole of total polymeric glucose) was found to have 6.9% end group (branch point content). The extent of labeling of the glucose units in outer chains of the glycogen was determined by allowing phosphorylase *a* to degrade 27% of the molecule to α-glucose-1-P in the presence of excess P_i. The residual limit dextrin was precipitated by 50% ethanol and washed once. The supernatant fluid and the washings were combined and the solution heated at 80° to evaporate most of the alcohol. The remaining solution was made 0.1 M in HCl and heated at 100° for 3 min to hydrolyze glucose-1-P to glucose. After cooling, the solution was deionized by passage over a column of Amberlite MB-3, and [^{14}C]glucose was separated from a small amount of residual ^{14}C limit dextrin by chromatography on Whatman No. 1 paper, using 1-butanol–pyridine–water (3:2:1.5) as the solvent. The eluted [^{14}C]glucose had a specific activity of 2660 cpm/μmole. The ^{14}C limit dextrin which had been isolated by ethanol precipitation was dialyzed exhaustively and its specific activity was 2870 cpm/μmole. Thus, the average specific activity of the outer 27% of the molecule, which is composed only of α-1,4-glucosidically linked units, was about equal to that of its branched core. Information on this point is important in view of the results

* From the Department of Biological Chemistry, Washington University School of Medicine, Saint Louis, Missouri 63110. *Received November 18, 1969.* Supported by Grant GM-04761 from the National Institutes of Health. Three preliminary reports have been published (Jeffrey *et al.*, 1967; Brown *et al.*, 1969; Jeffrey *et al.*, 1969).

† Present address: Department of Biochemistry, Monash University, Clayton (Victoria), Australia.

‡ Established Investigator of the American Heart Association.

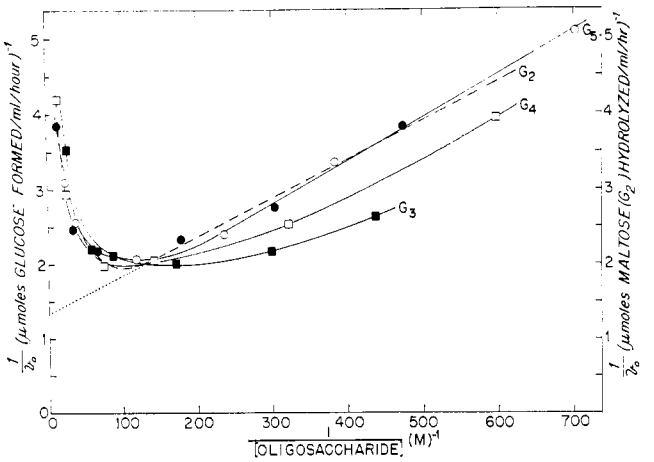

FIGURE 1: Dependence of rate of hydrolysis of maltosidically linked oligosaccharides on substrate concentration. Incubation was for 30 min at 37° in 0.04 M potassium acetate buffer (pH 4.1) containing 0.1 M KCl and 2.4 μg of protein/ml: (●--●) maltose (G_2); (■-■) maltotriose (G_3); (□-□) maltotetraose (G_4); (○-○) maltopentaose (G_5).

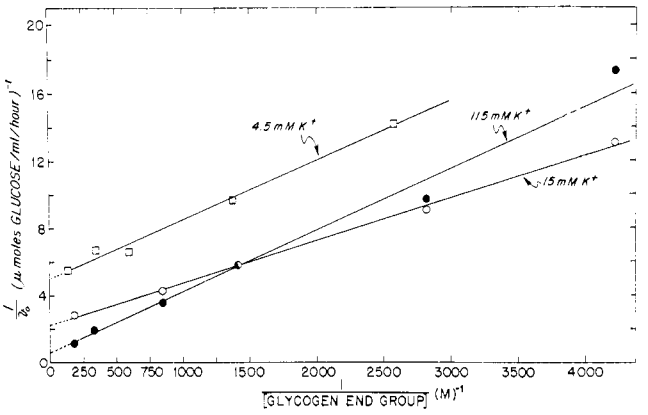

FIGURE 2: Dependence of rate of hydrolysis of glycogen on substrate concentration at different concentrations of KCl: (□-□) 8 mM potassium acetate–acetic acid buffer, pH 4.2; (○-○) 50 mM potassium acetate–acetic acid buffer, pH 4.2; (●-●) 50 mM potassium acetate–acetic acid buffer, pH 4.2, plus 0.1 M KCl. In every case incubation was for 20 min at 37° with 1.6 μg of protein/ml.

of kinetic studies reported below in which this [^{14}C]glycogen was used as a substrate.

Assays for α-1,4-glucosidase and α-1,6-glucosidase activities were done as described in the preceding paper (Jeffrey *et al.*, 1970).

Results

α-1,4-Glucosidase Action on Oligosaccharides of the Maltose Series. Maltose, maltotriose, maltotetraose, and maltopentaose were incubated separately with the purified glucosidase in pH 4.1 acetate buffer containing 0.1 M KCl, and the initial rate of glucose formation was measured as a function of substrate concentration. Figure 1 shows the results in the form of double-reciprocal plots. The velocity of glucosidase action on maltose is plotted as the micromoles of substrate hydrolyzed. In the case of each of the three higher linear homologs, the velocities are plotted simply as micromoles of glucose formed, since it is possible that the quantity of glucose measured initially may have included some which resulted from a rapid, unichain degradation of the substrate, with the result that more than 1 mole of glucose could have arisen from 1 mole of substrate. A detailed study of this point could only be made by chromatographic investigation of the quantity of each oligosaccharide intermediate present during the early stages of hydrolysis. The most striking feature of these curves is that they show a profound degree of substrate inhibition evident when the initial substrate concentration exceeds 5 to 10 mM. Extrapolation of the linear portion of the plot for maltose hydrolysis allows an estimate to be made that the K_m for this sugar is 3.8 mM. Substrate inhibition by maltose was observed in all kinetic experiments in which high concentrations of this substrate were used. The inhibition was evident whether or not KCl was present in the reaction mixture.

α-1,4-Glucosidase Action on Glycogen. Rabbit liver glycogen (end group or branch point content, 7.05%) was incubated with the purified glucosidase in potassium acetate–acetic acid buffers (pH 4.2) of several different concentrations and in the presence of various quantities of added KCl. The initial velocity of the formation of glucose was measured as a function of the concentration of glycogen. Figure 2 shows the results in the form of double-reciprocal plots of the data from three such experiments. Activation of the enzymatic reaction occurred in 50 mM acetate buffer containing 15 mM K^+ as compared with 8 mM acetate buffer containing 4.5 mM K^+. From the experimental results discussed in the preceding paper, it is most likely that this activation was due to increased cation concentration rather than to changes in the concentration of either acetate or chloride ion. It can be seen that increasing the cation concentration increases both the K_m of glycogen and the V_{max} of the glucosidase reaction. However, when the cation concentration is much higher (*e.g.*, 115 mM), the slope of the Lineweaver–Burk plot is changed such that the line becomes nearly parallel to that characteristic of the buffer system with the lowest K^+ concentration which was tested. Thus, at high glycogen concentration, the glucosidase reaction is activated in a progressive way as the concentration of K^+ is increased over a 25-fold range. However, at lower glycogen concentrations, the reaction velocity first increases with increasing cation concentration, but then, as the KCl concentration is increased still further, the reaction velocity decreases, although the reaction is still somewhat activated in 0.1 M KCl compared with its rate in a buffer containing only 4.5 mM KCl. This complex kinetic behavior was found consistently in experiments in which freshly prepared enzyme was used, as well as in others in which the glucosidase used had been kept frozen for several weeks and had lost 70% of its activity during this period of storage. When the activity of such an aged enzyme was tested in 5 mM K^+, the double-reciprocal plot of velocity *vs.* glycogen concentration was nonlinear at high polysaccharide concentration (figure 3B). However, it was found that the prominent downward curvature of the line could be abolished by increasing the KCl concentration as shown in the figure. These rather unusual observations cannot be explained fully by the kinetic data which it has been possible to obtain using the small amount of highly purified enzyme available, especially because of

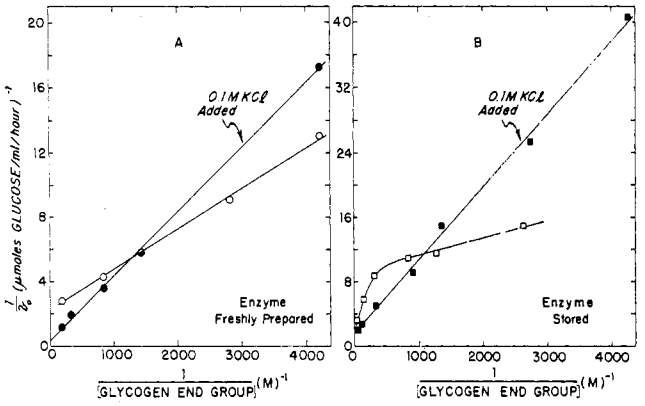

FIGURE 3: Effect of KCl on the kinetics of the action on glycogen of fresh and aged preparations of α-glucosidase. Part A: incubation was for 20 min at 37° in 0.05 M potassium acetate buffer, pH 4.2, with 1.6 μg of protein/ml. Part B: incubation was for 20 min at 37° in 8 mM potassium acetate buffer, pH 4.4, with 1.9 μg of protein/ml. For other details, see the text.

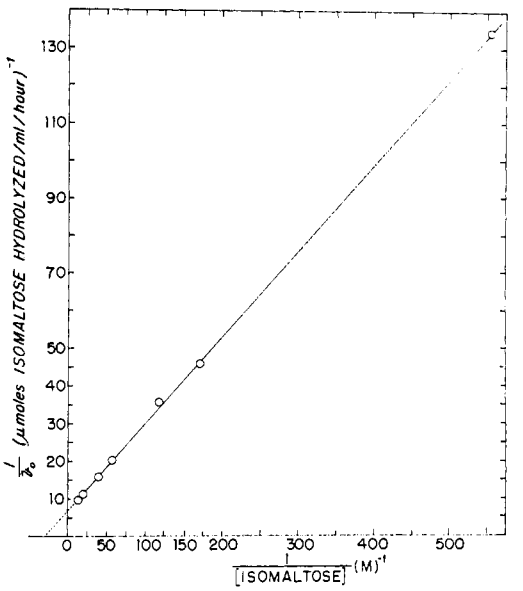

FIGURE 4: Dependence of rate of hydrolysis of isomaltose on substrate concentration. Incubation was for 65 min at 37° in 0.04 M potassium acetate buffer (pH 3.8) with 2.4 μg of protein/ml.

its instability upon storage. Whether the protein has a subunit structure has not been investigated, and the number of binding sites on the enzyme for glycogen is unknown. However, since the glucosidase has a molecular weight of about 114,000 (Jeffrey *et al.*, 1970), it is possible that it has a subunit structure and multiple binding sites for glycogen may be present. If this were the case, one explanation for a line with a downward curvature such as that shown in Figure 3B could be that negative cooperativity effects, dependent on cation concentration, exist in the binding of glycogen to the enzyme. Conway and Koshland (1968) have described the phenomenon of negative cooperativity in the binding of NAD^+ to glyceraldehyde 3-phosphate dehydrogenase. It should be emphasized, however, that the data presented here with regard to the kinetics of the action of the lysosomal glucosidase on glycogen are fragmentary, and a more extended discussion of possible explanations for them is not possible. Extrapolation of the straight lines in Figure 2 allows the estimate to be made that the V_{max} of α-1,4-glucosidase action on glycogen increases 8.9 times when the concentration of K^+ is increased from 4.5 to 115 mM at pH 4.2. At the same time, the K_m of glycogen increases from 0.7 to 6.5 mM, calculated as total outer chain end groups. When K^+ has an intermediate concentration of 15 mM, the K_m of glycogen is 1.1 mM. Although the K_m of maltose, as estimated by extrapolation of the linear portion of a Lineweaver–Burk plot (Figure 1), is 3.8 mM and, therefore, somewhat higher than that of glycogen at intermediate salt concentration, it is not possible to compare the two substrates in a meaningful way by this criterion, since the calculation for glycogen is made on the assumption that all of its outer chains are equally available for combination with the enzyme. This is probably not the case, since it has been found that different samples of liver glycogen have as much as a threefold difference in K_m expressed as total outer chain end-group concentration. The structural differences responsible for such variation are not known. However, in all cases, even at a concentration of 60 mg of polysaccharide/ml, there is no apparent substrate inhibition (Figure 3B). Thus, the macromolecular, polysaccharide substrate differs markedly from linear oligosaccharide substrates in the kinetics of its interaction with the enzyme.

α-1,6-Glucosidase Action on Isomaltose. Isomaltose was incubated with the purified glucosidase in acetate buffer (pH 3.8) containing no added KCl, and the initial rate of glucose formation was measured as a function of substrate concentration. Figure 4 shows the results in the form of a double reciprocal plot in which the velocity is expressed as the micromoles of substrate hydrolyzed per hour. Extrapolation of the data gives a K_m for isomaltose of 32.9 mM or about 8.5 times that of maltose. However, in contrast to the latter substance and to other linear, α-1,4-glucosidically linked oligosaccharides, isomaltose apparently does not show substrate inhibition, at least up to a concentration of 76 mM. Inhibition in the maltose series becomes apparent at concentrations above 8 mM (Figure 1). With respect to the lack of substrate inhibition, the action of the lysosomal glucosidase on the α-1,6-glucosidic bond of isomaltose is more like its action on the α-1,4-glucosidic bonds of glycogen than it is like that on oligosaccharides with maltosidic bonds. Since the enzyme had an action on both α-1,4- and α-1,6-glucosidic bonds, mutual inhibition studies were done using substrates having these different bond types. The results of such studies are discussed below.

Substrate Inhibition Studies. The effect of glycogen on the initial rate of hydrolysis of maltose and the effect of maltose on the action of the glucosidase on glycogen was studied by using one substrate labeled with ^{14}C and the other unlabeled. In this way, the quantity of [^{14}C]glucose produced from the first could be found by paper chromatography of the reaction mixture followed by elution of the glucose region and determination of the ^{14}C content of the eluate as described in the preceding paper. In one experiment, uniformly labeled [^{14}C]maltose (20,840 cpm/μmole) was incubated with the purified glucosidase in pH 4.0 acetate buffer. A second reaction mixture was similar in composition

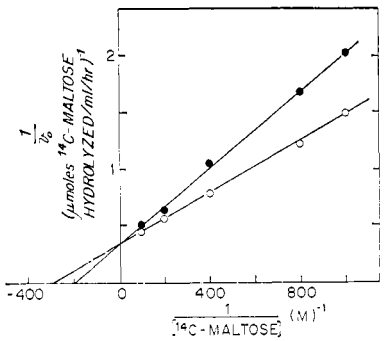

FIGURE 5: Kinetics of inhibition by glycogen of maltose hydrolysis. Incubation was for 20 min at 37° in 0.05 M potassium acetate buffer (pH 4.0): (O-O) no glycogen added; (●-●) 4.26 mM glycogen added (concentration calculated as total outer chain, nonreducing end groups). For other details see the text.

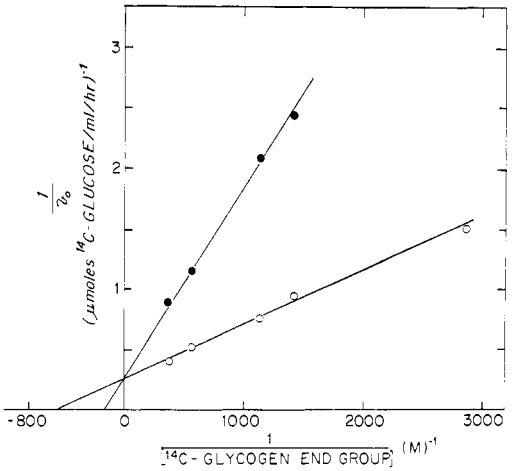

FIGURE 6: Kinetics of inhibition by maltose of α-1,4-glucosidase action on glycogen. Incubation was for 20 min at 37° in 0.05 M potassium acetate buffer (pH 4.2) containing 0.2 M KCl: (O-O) no maltose added; (●-●) 1.6 mM maltose added. For other details see the text.

except that it contained rabbit liver glycogen at a concentration of 4.26 mM expressed as total nonreducing end groups. In other experiments it had been shown that glucose formation in such a system was linear with time for more than 20 min. Hence, at the end of that period, the reaction mixtures were heated in boiling water for 1 min, and their [^{14}C]glucose content was determined as described. Suitable blank reaction mixtures incubated without enzyme, but otherwise treated identically, were carried through all of the chromatographic, elution, and counting procedures, and the very small amount of [^{14}C]glucose found therein was subtracted from that present in the corresponding complete reaction mixtures. Double-reciprocal plots of the velocity of [^{14}C]maltose hydrolysis in the absence and in the presence of glycogen are shown in Figure 5. It can be seen that glycogen appears to be a competitive inhibitor of maltose hydrolysis. The K_m for maltose was found to be 3.4 mM. If competition between these two substrates is assumed to be truly competitive, the K_I for glycogen is 8.6 mM with respect to outer chain end groups. This concentration is about eight times its K_m measured under the same conditions (Figure 2). Since the isotope experiment was carried out at only one concentration of glycogen, it is not possible to decide whether the two substrates are fully competitive or only partially so. In the latter case, the value calculated above for K_I would not be valid. It should also be noted that the experimental points of Figure 5 were obtained using concentrations of maltose which were less than those at which substrate inhibition is evident (*cf.* Figure 1). Thus, the experiment does not give information about the possible influence which glycogen might have on the binding of maltose at an inhibitor site on the enzyme, the presence of which seems likely in view of the data shown in Figure 1.

In another experiment [^{14}C]glycogen (prepared as described in Materials) was incubated at various concentrations with the purified glucosidase in pH 4.2 acetate buffer containing added 0.2 M KCl. A second reaction mixture contained, in addition, 1.6 mM maltose. Determination by the chromatographic procedure described above of the rate of formation of [^{14}C]glucose as a function of glycogen concentration gave data which are shown in Figure 6 in the form of double-reciprocal plots. The apparently competitive nature of maltose inhibition is clearly shown, but, in this case, as in the experiment shown in Figure 5, the use of only one concentration of inhibitor does not permit conclusive characterization of the inhibition as fully competitive. The data of Figure 6 give a K_m for the [^{14}C]glycogen of 1.7 mM with respect to outer chain end groups. This value is somewhat lower than that obtained (5.8 mM) in another experiment in which a different sample of rabbit liver glycogen was incubated with the glucosidase in the presence of 0.2 M KCl. It is probable that unknown features of the two polysaccharides may account for this difference. On the assumption that the inhibition by maltose is fully competitive, its K_I is 0.6 mM. This concentration is about one-sixth that of the K_m of maltose as determined in other experiments. Thus, a marked difference was found in both of these experiments between the K_m of each substrate and its apparent K_I when it acted as an inhibitor of the hydrolysis of the other. It has been shown that the hydrolysis of the two substrates is influenced differently by cation concentration, and also that the curves of velocity of hydrolysis as a function of pH for maltose and glycogen are dissimilar, although the formation of glucose from both is due initially only to α-1,4-glucosidase action. Taken together, these findings suggest that glycogen and maltose are not bound to the enzyme at the same catalytically active site. The results of the inhibition studies shown in Figures 5 and 6 suggest that two catalytically active binding sites may be involved, and that these are close enough together so that the binding of a substrate at one site can influence the binding of the other substrate at the second site under circumstances when both maltose and glycogen are present together.

An experiment was done to investigate the influence of isomaltose on maltose hydrolysis. Various concentrations of uniformly labeled [^{14}C]maltose (13,320 cpm/μmole) were incubated with the purified glucosidase in pH 4.0 acetate buffer. A second reaction mixture contained, in addition, 41.2 mM isomaltose. Determination of the rate of [^{14}C]glucose formation as a function of [^{14}C]maltose concentration gave data which are shown in Figure 7 in the form of double-reciprocal

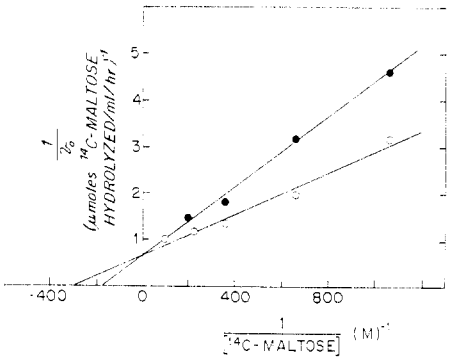

FIGURE 7: Kinetics of inhibition by isomaltose of maltose hydrolysis. Incubation was for 30 min at 37° in 0.05 M potassium acetate buffer (pH 4.0) with 1.9 μg of protein/ml: (O-O) no isomaltose added; (●-●) 41.2 mM isomaltose added. For other details, see the text.

plots. Although the data for the uninhibited system show considerable scatter, a straight-line fit by the least-squares method gave a K_m of 3.5 mM for maltose, and this value is in good agreement with other determinations (Figures 1 and 5). It can be seen from Figure 7 that isomaltose appears to be a competitive inhibitor of maltose hydrolysis. On the assumption that the inhibition is fully competitive, the K_I of isomaltose is 61 mM which is about two times the K_m of this substrate (*cf.* Figure 4).

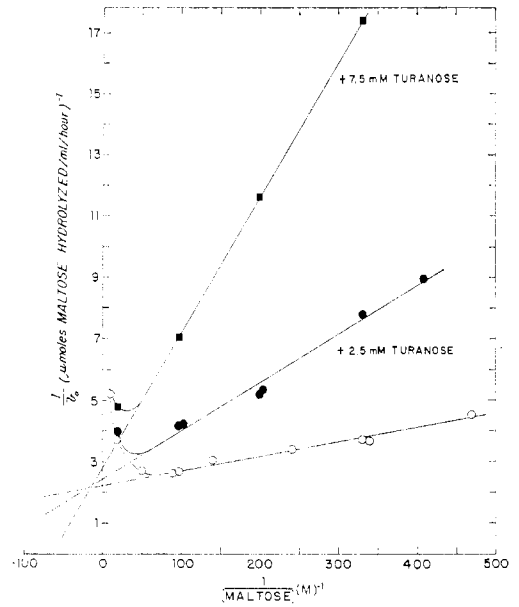

FIGURE 8: Kinetics of inhibition by turanose of maltose hydrolysis. Incubation was for 20 min at 37° in 0.04 M potassium acetate buffer (pH 3.7) with 1.6 μg of protein/ml: (O-O) no turanose added. All analyses for glucose were corrected by subtracting the small amount of this sugar which had been formed by the chemical (nonenzymatic) hydrolysis of turanose in the course of the experiment. This quantity was determined by measuring the amount of fructose, which had also been formed, by means of the addition of phosphoglucoseisomerase to the spectrophotometric coupled assay system (hexokinase plus glucose 6-phosphate dehydrogenase) used for glucose determination as described in Methods. The chemical hydrolysis of turanose yields equimolecular amounts of glucose and fructose.

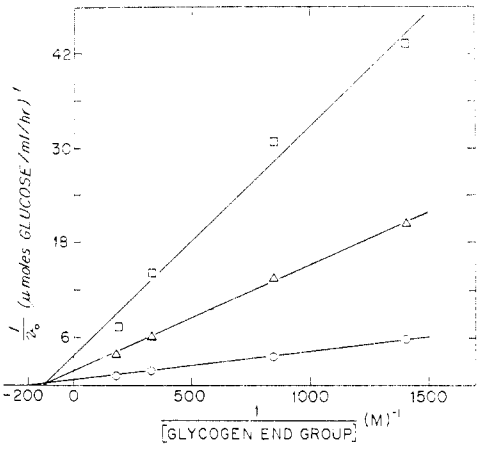

FIGURE 9: Kinetics of inhibition by turanose of α-1,4-glucosidase action on glycogen. Incubation was for 20 min at 37° in 0.05 M potassium acetate buffer (pH 4.2) containing 0.1 M KCl and 1.6 μg of protein/ml: (O-O) no turanose added; (△-△) 2.67 mM turanose added; (□-□) 6.67 mM turanose added. Corrections for the chemical hydrolysis of turanose were made as described in Figure 8.

Inhibition by Turanose. Lejeune *et al.* (1963) observed that, although the lysosome fraction of rat liver does not catalyze the hydrolysis of turanose, this disaccharide inhibits the maltase action of the lysosomal glucosidase. The inhibition was found to be noncompetitive. That turanose inhibits maltose hydrolysis at acid pH was confirmed by Auricchio and Bruni (1967) who used a more highly purified enzyme preparation from rat liver. These authors also showed that inhibition of glucose formation from glycogen at acid pH is strongly inhibited by the disaccharide. Bruni *et al.* (1969) have reported that turanose is a competitive inhibitor at pH 4.5 of the maltase action of an α-glucosidase purified from whole bovine liver. It was of interest to investigate the effect of turanose on the α-1,4-glucosidase and α-1,6-glucosidase activities of the rat liver lysosomal enzyme. The effect of two concentrations of turanose on maltose hydrolysis by the most highly purified enzyme is shown in Figure 8 as double-reciprocal plots of reaction velocity *vs.* maltose concentration. The inhibition by turanose appears to be of the mixed type. When plots are made according to Dixon (1953) of the reciprocal of the initial velocity *vs.* the turanose concentration for various concentrations of maltose, curved lines are obtained. These can be extrapolated only approximately, but from the point of their apparent intersection the K_I for turanose is estimated to be about 2.8 mM. A similar value was calculated from the data of Figure 8 on an assumption that the mixed inhibition by turanose is of a partially competitive plus a purely noncompetitive type. When the data of Figure 8 obtained in the absence of turanose are plotted according to Dixon (1953) with maltose concentration on the abscissa *vs.* the reciprocal of the initial velocity, the three points representing the highest substrate concentration define a straight line which can be extrapolated to the abscissa to give a K_I of 62 mM for maltose when it acts as an inhibitor of its own hydrolysis. An interesting point shown in Figure 8 is that substrate inhibition by maltose is evident even in the presence of a substantial concentration of turanose. It is possible that

TABLE I: Extent of Action of Lysosomal α-Glucosidase on Polysaccharides.[a]

Time of Incubation (hr)	Formation of Glucose (% of Total Polysaccharide)		
	Glycogen (4.3 mg/ml)	Phosphorylase Limit Dextrin (3.1 mg/ml)	β-Amylase Limit Dextrin (3.4 mg/ml)
2	35.7	31.0	26.7
4	45.8	42.1	36.7
7	50.8	54.3	48.7
24	91.2	94.5	84.9

[a] Rabbit liver glycogen (7.3% end group), a limit dextrin (11.7% end group) prepared from it by the action of phosphorylase, and a limit dextrin (14.5% end group) prepared by the action of β-amylase were incubated separately at 37° with 33 μg/ml of purified glucosidase in 0.07 M potassium acetate buffer, pH 4.2, containing added 0.2 M KCl. The formation of glucose was determined as described in Methods. After 8 hr, the enzyme concentration was increased to 77 μg/ml by the addition of fresh enzyme, and incubation was continued overnight under toluene vapor.

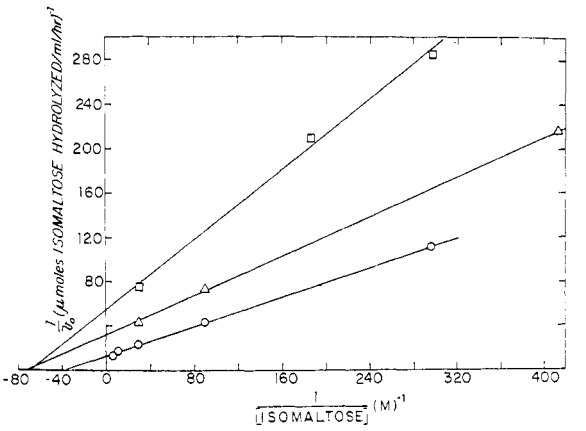

FIGURE 10: Kinetics of inhibition by turanose of isomaltose hydrolysis. Incubation was for 60 min at 37° in 0.04 M potassium acetate buffer (pH 4.2) with 2.4 μg of protein/ml: (O-O) no turanose added; (Δ-Δ) 2.5 mM turanose added; (□-□) 5.0 mM turanose added. Corrections for the chemical hydrolysis of turanose were made as described in Figure 8.

mixed inhibition by turanose is attributable to its competition with the substrate maltose for an inhibitory site on the enzyme which can be occupied by either of these disaccharides.

When turanose inhibition of the α-1,4-glucosidase action on glycogen was studied in 0.1 M KCl, the data shown in Figure 9 were obtained. Although the inhibition might appear to be noncompetitive, careful study of this point in this and in several other experiments showed that the inhibition is really of the mixed type. Thus, in Figure 9, the K_m for glycogen is 6.4 mM. In the presence of 2.7 mM turanose, the apparent K_m becomes 7.9 mM, and in the presence of 6.7 mM turanose, the apparent K_m for glycogen is 8.0 mM. Preliminary experiments have suggested that in the presence of KCl, the tendency of turanose to give mixed inhibition of glycogen hydrolysis is depressed, so that in high salt concentration the effect of the disaccharide becomes nearly that of a purely noncompetitive inhibitor.

The inhibitory effect of turanose on the α-1,6-glucosidase action of the enzyme is shown in Figure 10 with isomaltose as the substrate. The double-reciprocal plot of data obtained in the presence of 2.5 mM turanose is nearly parallel to that obtained in the absence of any inhibitor. When the turanose concentration was increased to 5 mM, the plot becomes almost typical of purely noncompetitive inhibition. These data may indicate that turanose very strongly affects the rate of breakdown of the enzyme–substrate complex into glucose, and that with isomaltose as substrate, the rate constant for this breakdown makes an especially important contribution to the numerical value of the Michaelis constant of the substrate determined kinetically. It may be recalled that the K_m of isomaltose was found to be about 8 times larger than that of maltose. It is possible that the marked differences in the effect of turanose on the hydrolysis of maltose (Figure 8) and of isomaltose (Figure 10) are due, in part, to differences in the relative contribution to the value of K_m made by the rate constant for the decomposition of the enzyme–substrate complex formed by combination of the enzyme with each of these disaccharides of different bond type.

Extent of Action on Polysaccharides. In view of the finding that isomaltose is a substrate for the lysosomal glucosidase, it was of interest to find whether the enzyme preparation was able to catalyze the total degradation of glycogen to glucose. Such a degradation would be possible only if the α-1,6-glucosidic linkages at the branch points of the polysaccharides were susceptible to enzymic hydrolysis. Table I shows the results of an experiment in which glycogen was incubated with the enzyme at the pH and salt concentration which had been found to be optimal for assay of the α-1,4-glucosidase activity using this polysaccharide as the substrate. The extent of glucose formation measured at several times showed clearly that debranching of the glycogen had indeed occurred. That debranching of other polysaccharides could occur also was shown by the use of limit dextrins prepared from glycogen. A dextrin prepared by β-amylase action is known to have only one or two maltosidically linked glucose units peripheral to the α-1,6-glucosidically linked unit at each outer branch point. A limit dextrin prepared by the action of phosphorylase on the average has three such units. The rate and extent of glucose formation from these substances (Table I) demonstrates the potentiality of the enzyme preparation for bringing about the nearly total hydrolytic degradation of branched polysaccharides derived from glycogen.

It would be of interest to know the rate at which debranching of glycogen by the enzyme can proceed compared with the rate of hydrolysis of its maltosidically linked outer chain units. This can not be determined satisfactorily by direct measurement because of the unavailability of a limit dextrin model compound whose structure is that of the intermediate product existing just prior to the debranching

reaction catalyzed by the glucosidase. However, this question was studied by allowing the purified rat liver glucosidase to act on glycogen isolated from heart muscle removed at autopsy from a patient with type II glycogen storage disease. The absence in this patient's heart of any glucosidase active on glycogen at acid pH had been shown previously by direct assay of the muscle tissue. After the rat liver enzyme had been allowed to convert 80% of this human glycogen sample into glucose, the residual polysaccharide was isolated quantitatively and its structure was studied. One-quarter of this residual substance was totally resistant to degradation by the combined action of phosphorylase and the debranching enzyme system, amylo-1,6-glucosidase–oligo-1,4→1,4-glucan transferase. This fact suggests that in this fraction there may have been only a single glucose unit in α-1,4-glycosidic linkage covering each outermost branch point unit and thus preventing hydrolysis of the branch point by amylo-1,6-glucosidase. The remainder of the polysaccharide was degradable and was found to have a branch point content of 14.5%. This value is greater than that characteristic of a phosphorylase limit dextrin of glycogen. Accordingly, it is probable that in most of this latter fraction, the polysaccharide's external side chains had fewer than three maltosidically linked glucose units covering each α-1,6-glucosidically linked unit at the outer branch points. Taken together these results indicate that lysosomal glucosidase action on glycogen *in vitro* becomes relatively slow as branch points are approached. However, the experiment does not provide any information about the kinetic factors responsible for this effect.

Discussion

The results of the studies reported in this paper show that the α-glucosidase purified from rat liver lysosomes has complex kinetic behavior. Most of the data which have been obtained are consistent with the hypothesis that the enzyme may have at least two catalytically active binding sites which are relatively close together and which, thus, may interact with each other. One of the two catalytic sites can bind the disaccharide substrate, maltose, as well, perhaps, as other maltosidically linked oligosaccharides of low molecular weight. However, no direct information is available on this latter point. This same binding site also seems to have affinity for the α-1,6-glucosidically linked disaccharide, isomaltose, since this substance is a competitive inhibitor of maltose hydrolysis. The other catalytically active site(s) may bind polysaccharide substrates such as glycogen and would be the locus of α-1,4-glucosidase action on such molecules. Although limited data suggest that maltose and glycogen seem to be mutually competitive substrates, there are large differences between the K_m of each and its apparent K_I as an inhibitor of the hydrolysis of the other. Thus, the K_I for glycogen is about eight times its K_m, while the K_m for maltose is about six times its K_I. It has been shown in the preceding paper (Jeffrey *et al.*, 1970a) that the initial velocities of hydrolysis of these two substrates are influenced differently by cation concentration and by pH changes. If two or more catalytically active binding sites were assumed to interact with each other, the observed influence of one substrate on the K_m of the other, when both are present together, might be partially explained.

In addition to the substrate binding site(s), there seems to be an inhibitory site on the enzyme at which maltose and other low molecular weight oligosaccharides of glucose having α-1,4-glycosidic bonds can be bound. Polysaccharides such as glycogen and the α-1,6-glucosidically linked disaccharide, isomaltose, have little or no affinity for this site. It is probable that turanose, 3-*O*-(α-D-glucopyranosyl)-D-fructose, also has some affinity for this inhibitory site, as well, perhaps, as for the catalytically active site where the oligosaccharide substrates are hydrolyzed. The fact that turanose is almost a purely noncompetitive inhibitor of glycogen hydrolysis suggests that it has little or no affinity for the glycogen binding site. The suggestions which have been made here regarding the nature of the various binding sites of the enzyme are susceptible to future experimental study in the light of the possibility that this lysosomal enzyme, whose molecular weight is 114,000, may, in fact, be found to have a subunit structure.

It has been found that the purified lysosomal α-glucosidase is able to catalyze the total hydrolysis of glycogen to glucose under suitable conditions *in vitro*. The debranching of the polysaccharide by hydrolytic cleavage of its α-1,6-glycosidically linked branch point units is the rate-limiting step in the overall degradation of glycogen. However, it is unknown whether these relative rates of chain shortening and of debranching of the polysaccharide have any metabolic significance in controlling the rate of glycogen catabolism within the lysosome.

The following paper (Brown *et al.*, 1970) shows that the α-1,4-glucosidase and α-1,6-glucosidase activities at acid pH are both missing from the tissues of patients who have the congenital disease known as type II glycogenosis.

References

Auricchio, F., and Bruni, C. B. (1967), *Biochem. J. 105*, 35.

Brown, B. I., Brown, D. H., and Jeffrey, P. L. (1970), *Biochemistry 9*, 1423.

Brown, D. H., Jeffrey, P. L., and Brown, B. I. (1969), *Abstr. 6th Meeting FEBS Madrid*, 283.

Bruni, C. B., Auricchio, F., and Covelli, I. (1969), *J. Biol. Chem. 244*, 4735.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Jeffrey, P. L., Brown, B. I., and Brown, D. H. (1967), *Fed. Proc. 26*, 345.

Jeffrey, P. L., Brown, B. I., and Brown, D. H. (1969), *Proc. Aust. Biochem. Soc.*, 7.

Jeffrey, P. L., Brown, D. H., and Brown, B. I. (1970), *Biochemistry 9*, 1403.

Lejeune, N., Thinès-Sempoux, D., and Hers, H. G. (1963), *Biochem. J. 86*, 16.